TOXIC EFFECTS OF NORMAL SERA AND HOMOLOGOUS ANTISERA ON THE CHICK EMBRYO[1]

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The possibility of identifying embryonic antigens of unique function, of localizing their sites of origin and action, and of manipulating them experimentally in order to analyze their developmental significance by the use of specific toxic sera at lethal or sub-lethal doses was advanced by Nace (1955). By modifying the normal function of an antigen with sub-lethal doses of a toxic antiserum, a specific anomaly may be produced, affording a key to the localization and the time and nature of the action of the antigen. Similar arguments have been advanced by those who have sought to block the growth of tumors with specific antisera (reviewed by Ross, 1957; Wissler and Flax, 1957). However, before this approach can be employed critically in studying the synthesis of specific antigens and their role in development, the following questions must be considered: (1) Are the proteins and other macromolecules of the embryo antigenic? Or does the embryo contain a population of molecules capable of reacting with antibody produced against adult antigens but incapable of eliciting antibody production? The distinction must be made between the occurrence in embryos of combining groups identical with those of adult antigens and the occurrence of embryonic antigens (Ebert, 1958a). (2) What are the effects of antisera on the embryo? Does the reaction between antigen and antibody, *in vivo*, result in measurable modifications of, or interference with, biological function? As a general rule, tissue-specific molecules exhibit species-specificity to some degree, making analysis by immunochemical techniques possible; the principal advantage of these methods is their exquisite sensitivity, which makes possible the analysis of the rate of synthesis and accumulation and site of localization of proteins or other macromolecules present in embryos in trace amounts. The principal difficulty, one which is often not appreciated, is that antigenic specificity depends upon relatively small determinant groups rather than on the complete structure of the molecule, and that the molecule may contain more than one kind of determinant group. Little is known of the kind, number, and size of determinant groups of natural proteins. The antigenically active groups and physiologically active groups of a molecule may not be

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identical. Similarity in immunochemical properties of natural molecules of diverse origin may result from (1) identity of one or more antigenic determinant groups, (2) a degree of structural similarity, or (3) the presence of one as an undetected trace contaminant in the other, for, inasmuch as traces of antigen may evoke large amounts of antibody, such contaminants tend to be revealed (Ebert, 1958b).

The antigenicity of embryonic tissues has been demonstrated in several species, for example in amphibians (Cooper, 1948, 1950; Flickinger and Nace, 1952; Clayton, 1953) and in the sea urchin (Perlmann, 1954; Perlmann and Perlmann, 1957). Much of the earlier literature has been reviewed by Tyler (1955, 1957).

The ability of the chick embryo to react with antisera against tissue-specific components of adult chickens was shown by Burke *et al.* (1944), Nace and Schechtman (1948), Ebert (1950, 1951), and Ebert *et al.* (1955). For example, specific effects of antibodies against adult antigens on the development of chick embryos were reported by Ebert (1950). Saline extracts of heart, brain, and spleen from adult chickens were used as antigens. Rabbit antiserum against chicken brain affected chiefly nervous tissue, and anti-heart and anti-spleen sera affected primarily mesodermal elements in early chick blastoderms cultivated *in vitro*. Moreover, comparing the effects of anti-heart and anti-spleen preparations, it was clear that the former antisera affected the development of the heart, whereas the latter did not. A striking extension of this approach is found in the report by Langman *et al.* (1957) who have demonstrated specific effects of antisera developed in rabbits against antigens of chick lens, purified alpha crystallin, and myosin. Anti-lens and anti-alpha crystallin sera prevented the formation of the lens from ectoderm-optic cup combinations *in vitro*, whereas anti-myosin sera permitted normal lens development but inhibited mesenchyme formation. These studies clearly demonstrate the existence of reactive or combining groups in the embryo capable of reacting with antibodies to adult tissue antigens. However, the antigenicity of the chick embryo, *i.e.*, its ability to elicit the production of precipitating antibodies in the rabbit, cannot be inferred from these studies.

After complete absorption with adult laying hen serum, antisera against the serum of the 10-day chick embryo showed positive precipitating activity with the homologous antigens (Schechtman *et al.*, 1954). Moreover, Levi and Schechtman (1954) concluded from similar studies that the 12-day embryo contained distinct embryonic red blood cell antigens. Nettleship (1953) injected 1-day, 2-day, and 6-day chick embryos (p. 325), "emulsified in normal saline by syringe suction and expulsion or grinding in a mortar without abrasive," into hamsters. The hamster anti-chick sera were dropped or injected into or near the "embryo site" of unincubated eggs. The titers of the antisera used were not determined, nor were the antisera against the various stages tested with the homologous embryos. The hamster anti-chick embryo serum (p. 326) "placed in proximity to the preincubated chick embryo stops the development of these embryos at a time which corresponds to the time the embryo antigen was obtained." The results were interpreted as pointing strongly (p. 327) "to the development of qualitatively different protein complexes in the embryo concurrent with the embryo's growth." These results are consistent with the studies of Cooper (1950), Spar (1953), Nace (1953), and Flickinger and Nace (1952) showing changes in the antigenic constitution of the developing embryo. However, critical absorption techniques, such as those of Cooper (1950) and Spar (1953) in which antisera to later stages

were absorbed with antigens of the earlier stage, thus separating those antibodies peculiar to the later stages, were not reported. Tyler (1957) remarked (p. 356) that "For an unabsorbed antiserum of this type to produce a highly specific effect does seem surprising, and one wonders whether or not this might be due to fortuitous variation in the antibody content of the various antisera."

The present experiments were undertaken with two-fold objectives: (1) To establish whether or not the chick embryo contains specific antigens (as opposed to combining groups capable of reacting with antibodies to adult tissues) by injecting whole chick embryos or embryo extracts into rabbits, and (2) to examine the effects of the antisera thus produced on the development of the homologous embryo. However, because fresh rabbit serum was found to be highly toxic to the chick, it became necessary first to study the known heat-labile and heat-stable factors in fresh rabbit serum in order to devise means of reducing or removing false reactions, after which the action of antisera could be explored.

Materials and Methods

Preparation of antigens

The 72-hour chick embryo (stages 16 to 18, Hamburger and Hamilton, 1951) was used for the preparation of antigens because of the significant number of well defined histogenetic and morphogenetic processes which occur during this period, *e.g.*, morphogenesis and the growth of the limb buds, the deposition of the pigment in the eyes. Another factor in selecting the 72-hour embryo was its larger size and higher content of protein nitrogen as compared with that of the 48-hour embryo. Even in using the 72-hour embryo, a substantial number of embryos was required for the preparation of the antigens. For example, it required approximately twenty 72-hour embryos to furnish material for one injection into a single rabbit.

The embryos were cut from the yolk and transferred immediately into a dish containing ice cold 0.15 *M* NaCl. The adhering yolk was trimmed off with sharpened steel needles, and all membranes were removed with sharpened jewelers forceps. The embryos were rinsed several times in ice cold saline and stored in the freezer (− 20° C.) until used.

Saline extracts of the 72-hour embryo were prepared by permitting the frozen embryos to thaw at room temperature, after which they were homogenized with ice cold saline in a chilled Ten Broeck grinder. Approximately 1 ml. of saline was added for each 5 embryos. The cloudy suspension was refrigerated for 10 to 12 hours, after which it was centrifuged at 3000 RPM (1200 RCF) for 30 minutes at 0 to 4° C. The protein nitrogen content of the resultant translucent extract was approximately 0.2 mgN/ml., as determined by semi-micro Kjeldahl method.

Preparation of antibodies

In preliminary experiments, 6 white rabbits weighing 2 to 3 kilograms were injected intravenously with the saline extract and intraperitoneally with homogenized 72-hour chick embryos. Although injections and booster shots were given repeatedly, antisera with workable titers were not obtained. In subsequent experi-

ments, 10 rabbits were injected with antigens in adjuvant; 72-hour chick embryos and equal amounts of Falba, paraffin oil and heat-killed tubercle bacilli were ground together in a mortar without abrasive and emulsified by syringe suction and expulsion. Each rabbit received approximately 10 to 12 embryos (at least 10 mg. of protein) in each of three injections administered subcutaneously in the region of the neck, one week apart. The rabbits were bled from the marginal vein of the ear one month after the first injection and one week after an intravenous booster shot of 10 to 20 embryos homogenized in a small amount of saline. This method yielded antibodies with titers of 1:32 to 1:128. By standards conventionally employed for antibodies against purified antigens, these titers are low. As will be made clear, however, they proved to be valuable tools.

Tests of antibody content

The presence of antibodies was detected by the use of interfacial "ring" tests in which 0.1 ml. of the test serum was overlaid with 0.1 ml. of the serially diluted chick embryo extract. The tests were performed in 6 × 50 mm. culture tubes and test materials were delivered with measuring pipettes ground and fitted with hypodermic needles at the tip. After the appearance of the rings was noted, the tubes were mixed and placed in the refrigerator overnight. The next morning the tubes were tapped gently and the presence of precipitate was detected as a thin white spiral rising from the bottom of the tube.

The titer of the antiserum used was determined by precipitin tests in which 0.1 ml. of the embryo extract was added to 1 ml. of the serially diluted antiserum. The tubes were then incubated in a water bath at 37° C. for 30 minutes. The tubes were read after incubation and again after being refrigerated overnight.

Operative procedures

A technique for the study of the effects of antisera on the chick embryo was described by Witebsky and Neter (1935), who added serum drop by drop to the embryo. A modification of this technique was adopted in this study. New Hampshire eggs obtained from a local hatchery were incubated for 72 hours at 37.5 to 38° C. Preparatory to operation, the egg was swabbed with 70% alcohol. Then a square window 1 cm. × 1 cm. was cut in the shell with a sharpened hack saw blade. The cut piece of shell was removed, and the shell membrane was cut off. After the embryo was in position immediately under the window, a small hole was made in the vitelline membrane just anterior to the heart, after which 0.05 to 0.1 ml. of the test serum was inserted. The material, especially if colored and dense, could be seen to envelop the embryo and remain in position for several hours. The window was sealed with cellophane tape, and the egg was returned to the incubator.

All test materials were sterilized by autoclaving or by Seitz filtration. After the latter procedure the titer of each serum was checked because of the report (Dilks and Wolfe, 1949) that significant decreases in titer result from Seitz filtration. In the present study, decreases in titer were minimized by filtering large volumes of undiluted serum.

More than 50 experiments were conducted. Each experiment consisted of at least 25 to 35 embryos, including sham-operated or saline controls, and normal or

absorbed serum controls. Each embryo was numbered; the time of operation, the stage of the embryo, and the amount of the test substance administered recorded. All observations were recorded following examination of the specimens under a binocular dissecting microscope, after which the embryos were removed and dissected or fixed in calcium formol for further histological studies.

In the interest of objectivity, frequently the assistance of a second person was enlisted to code the randomly numbered treated eggs and to record the observations made by the experimenter.

The Effects of Normal Rabbit Serum on the 72-Hour Chick Embryo

In preliminary experiments, it was found that sera from both uninjected and injected rabbits were toxic to the embryo. Within a few minutes after the application of fresh rabbit serum the blastoderm begins to shrivel, the embryo gradually sinks and the heart stops beating. The toxicity of fresh rabbit serum has been encountered by others. Witebsky and Neter (1935) described its effects on the chick embryo. Bernheimer and Harrison (1940) observed the ability of normal rabbit serum to immobilize *Paramecium*. Green (1946) observed that normal

Table I

The effect of heating on the toxicity of fresh normal rabbit serum (NRS)

Serum	No. embryos treated	Effects on 72-hour chick embryos		
		Normal	Abnormal	Dead
Unheated NRS	117	3	3	111
NRS heated at 37° C. 30 minutes	8	0	0	8
NRS heated at 42° C. 30 minutes	8	1	0	7
NRS heated at 50° C. 30 minutes	10	5	0	5
NRS heated at 56° C. 30 minutes	17	17	0	0

rabbit serum interfered with the rapid growth of cancer cells, and Imagawa *et al.* (1954) observed that normal rabbit serum inhibited the proliferative capacity of mouse mammary cancer cells. A spermicidal factor in fresh human, bovine, rabbit, and rat sera was reported by Chang (1947). Nace has described normal rabbit sera which were toxic to the *Rana pipiens* embryo (1955; see also Nace and Inoue, 1957).

Witebsky and Neter (1935) reported that the toxic effects of fresh rabbit serum were removed by heating at 56° C. for 30 minutes, an observation confirmed in the present study. Partial inactivation was obtained by heating at 50° C. for 30 minutes, but below 50° C. inactivation did not occur (Table I). What is the nature of the heat-labile substance? Is it *complement* which is defined in part on the basis of its destruction by heating at 56° C. for 30 minutes? Is it *properdin*, the heat-labile substance recently found in normal serum of a number of animals (Pillemer *et al.*, 1954), or is it another heat-labile substance as yet undescribed? Other questions may be asked, among them: does the heat-labile factor act independently, or does it require the presence of heat-stable and/or other heat-labile factors for its action? What is the mechanism of its action?

TABLE II

The toxicity of heated normal rabbit serum (HNRS) coupled with unheated chicken serum (CS) or unheated guinea pig serum (GPS)

Serum	No. embryos treated	Effects on embryos		
		Normal	Abnormal	Dead
HNRS	43	43	0	0
CS	29	26	0	3
HNRS + CS (10 to 50%)	11	11	0	0
GPS	40	38	2	0
HNRS + GPS (10 to 50%)	27	26	1	0

The role of complement in the toxicity of fresh rabbit serum was examined first. The sufficiency of complement was tested by adding complement in the form of fresh guinea pig serum or fresh chicken serum to heated normal rabbit serum. The toxicity which was characteristic of fresh rabbit serum was not restored to heated rabbit serum by the addition of either fresh chicken serum pooled from 4 to 6 chickens, or fresh guinea pig serum pooled from 4 to 6 guinea pigs (Table II).

Next, complement or components of complement were removed from rabbit serum by absorbing unheated fresh rabbit serum with a nonspecific precipitate which had been prepared by combining beef serum albumin (BSA) with heated homologous rabbit antiserum (anti-BSA). The precipitate was washed three times with cold saline, after which 10 ml. of fresh normal rabbit serum were added to 0.5 ml. of packed precipitate. The mixture was refrigerated (0 to 4° C.) for 8 to 12 hours with frequent stirring. The mixture was centrifuged; next the supernatant was poured into another tube containing 0.5 ml. of packed beef serum albumin precipitate. After three absorptions, the rabbit serum was unable to lyse chicken red blood cells. The fresh rabbit serum absorbed in this manner was still toxic to the 72-hour chick embryo. The toxicity was lost only after heating for 30 minutes at 56° C. (Table III). These experiments clearly demonstrate that the heat-labile substance, complement, which can be absorbed by a nonspecific precipitate, is neither sufficient nor necessary for the toxic action of fresh rabbit serum.

TABLE III

The toxicity of normal rabbit serum (NRS) absorbed in the cold with beef serum albumin (BSA) precipitate and chicken red blood cells (RBC)

Serum	Absorbed with	No. embryos treated	Effects on embryos		
			Normal	Abnormal	Dead
NRS	BSA	70	37	5	28
NRS	BSA then heated	33	32	0	1
NRS	RBC	36	6	3	27
NRS	RBC then heated	30	28	2	0
NRS	BSA and RBC	16	6	4	6
NRS	BSA and RBC then heated	10	10	0	0

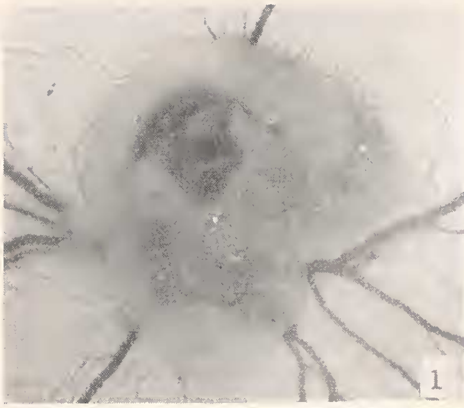
To determine whether or not heat-labile factors were capable of acting independently, heat-stable substances found in normal rabbit serum were removed by absorption. Several substances can be used for absorption purposes, among them, chicken red blood cells which were selected because they contain Forssman antigens (Boyd, 1956). In this manner, Forssman antibodies, as well as other substances absorbable by chicken red blood cells, can be removed. To minimize the destruction of heat-labile substances during the absorption process, the procedure was conducted in the cold (0 to 4° C.). Red blood cells were obtained from the pooled blood of 4 to 6 adult New Hampshire chickens. Approximately 2 ml. of packed red blood cells were used in the absorption of each 10 ml. of serum. The red blood cells and serum were thoroughly mixed by frequent stirring. After 8 to 12 hours, the cells were removed by centrifugation at 1200 RCF for 30 minutes. The serum was poured into another tube containing 2 ml. of packed red blood cells. The process was repeated until no further agglutination of red blood cells was observed under the microscope. The cold-absorbed rabbit serum was still highly toxic to the 72-hour chick embryo.

Since lysis frequently occurred during the long course of absorption in the cold, it was necessary to inactivate complement by removing cations before absorbing with red blood cells either by filtering the serum through a column of cation exchange resin (IRC-50, Rohm and Haas Company, Philadelphia, Pa.), as described by Levine *et al.* (1953), or by adding Versene (sodium ethylene diamine tetraacetate) to the serum. The latter method was found to be more successful. Upon completion of absorption, calcium and magnesium ions were reconstituted to a final concentration of 0.00015 *M* and 0.0005 *M*, respectively (Mayer and Levine, 1954).

The cold-absorbed serum which contained Versene and an insufficient amount of calcium and magnesium ions was found to be toxic to the 72-hour chick embryo. The picture of toxicity, however, differed from that produced by fresh rabbit serum. Upon the injection of absorbed serum containing Versene (a 6 millimolar solution of Versene in serum), the embryo dies within a few minutes. The heart is engorged with blood and becomes bright red in appearance. However, the puckering of the blastoderm and the sinking of the embryo, which is characteristic of the effect of fresh rabbit serum, is not observed. The toxic effects of Versene are observed only when insufficient calcium and magnesium ions are present.

To determine whether or not complement was still present in the cold-absorbed rabbit serum, its ability to lyse chicken red blood cells was tested. The cold-absorbed serum was unable to lyse chicken red blood cells. However, when a sufficient amount of heated but unabsorbed rabbit serum was added (1 : 1), lysis occurred readily. Thus, complement, which is dependent on the presence of heat-stable substances, was not destroyed in the process of absorption in the cold. Again, the toxic effects of cold-absorbed rabbit serum can be removed by heating (Table III). This result demonstrates the presence of a toxic heat-labile substance in fresh rabbit serum, a substance not absorbed by chicken red blood cells.

Absorption of fresh rabbit serum with beef serum albumin precipitates followed by chicken red blood cells in the cold also failed to remove the toxicity of the serum (Table III). It thus may be concluded that complement is neither sufficient nor necessary for the toxic action of fresh normal rabbit serum which is evoked in the absence of substances absorbable with chicken red blood cells. Moreover, since



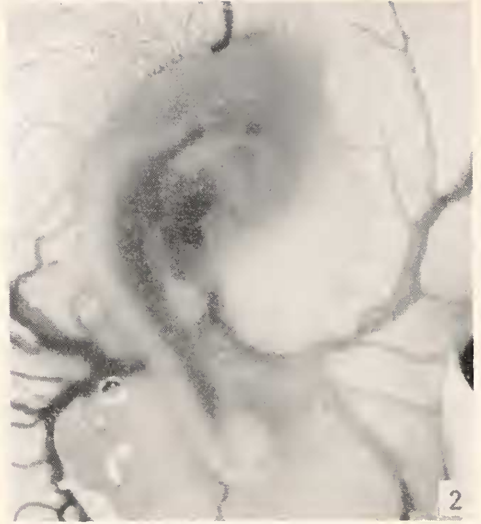


Plate I

Photographs were taken *in ovo* through the cut window 20–24 hours after treatment.

the action of properdin requires both complement and magnesium ions (Pillemer *et al.*, 1954), it is suggested that this heat-labile substance is not properdin.

The Effects of Antisera Against the 72-Hour Chick Embryo on the Homologous Embryo

It is clear from the foregoing experiments that to remove nonspecific toxic factors, normal rabbit sera and antisera must be heated for 30 minutes at 56° C. When heated rabbit antiserum against the 72-hour chick embryo was placed on the embryo, the immediate puckering of the blastoderm, together with its sinking, which was characteristic of fresh rabbit serum, was not observed. However, a number of the embryos died after 6 to 8 hours; in some cases the embryos did not show any visible effects until 15 to 18 hours after the operation, at which time slight abnormalities were detected. Usually no further changes appeared in the surviving embryos after 18 to 20 hours. Occasionally, some of the embryos with

Table IV

The effects of heated rabbit antiserum against the 72-hour chick embryo (HA72) coupled with guinea pig serum (GPS)

Serum	No. embryos treated	Graded effects on 72-hour chick embryos				
		1	2	3	4	5
HNRS	60	59	1	0	0	0
A72	51	1	0	0	0	50
HA72	57	28	7	6	16	0
HNRS + GPS	27	25	1	1	0	0
HA72 + GPS	69	19	3	8	6	33

slight visible abnormalities appeared to recover completely. The toxic effects of the sera on the chick embryo arbitrarily are divided into five different groups (Table IV; Plate I).

Plate I

Group 1. The embryos appear essentially normal with good colcr, as compared with unoperated embryos of the same stage (Fig. 1).

Group 2. The embryos appear essentially normal in stage and color but show slight morphological abnormalities, *e.g.*, the trunk may be turned ventrad, instead of to the left in embryos in stage 22. These abnormalities may be detected 15 to 18 hours after the operation (Fig. 2).

Figure 1. A group 1 embryo which is alive and appears normal (6 ×).

Figure 2. A group 2 embryo with accumulation of blood in trunk region (6 ×).

Fig. 3. A group 3 embryo which is alive with its head beneath the puckered portion of the blastoderm (6 ×).

Figure 4. A group 4 embryo which is dead. The embryo lies on top of the blastoderm which is smooth in appearance (6 ×).

Figure 5. A group 5 embryo which is dead and partially hidden by the puckered blastoderm (6 ×).

Group 3. The embryos are alive but show distinct abnormalities, *e.g.,* the trunk may be turned to the left or even doubled back upon itself. The embryos are usually pale in color. These abnormalities may be detected 10 to 12 hours after operation (Fig. 3).

Group 4. The embryos are dead and appear quite small and shrunken; blood vessels are not distinct. These embryos usually die 5 to 8 hours after the operation (Fig. 4).

Group 5. The embryos are dead. The blastoderm appears puckered or pursed. The blastoderm may be seen to begin to shrivel 3 to 5 minutes after the operation. The red blood cells may be seen to clump in the blood vessels in a few minutes and then cease to flow in the smaller vessels. The heart may stop beating as soon as 5 minutes after the operation (Fig. 5).

This classification of the extent of the toxic action on the embryo does not imply the expression of basically different mechanisms or functions in each of the five groups, nor does it indicate distinct and separate stages or steps of a single mechanism or function. The embryos earlier described as "normal" fall into either group 1 or 2. Embryos described as "abnormal" are similar to those in group 3, whereas embryos described as "dead" fall into either group 4 or 5. The low sensitivity of the system, probably owing in part to the low titer of the antisera employed (1 : 32 to 1 : 128), as well as to the heating of the antisera, increased the possibility of introducing false negative reactions. Methods were sought, therefore, to increase the sensitivity of the system. From the foregoing discussion, it is apparent that methods to achieve this end are available; *viz.*, the expedient of adding back those substances which are destroyed by heating, such as complement and properdin, but which do not contribute to the toxicity of normal rabbit serum.

The Effects of Guinea Pig and Rat Serum on the Activity of Rabbit Antiserum Against the 72-Hour Chick Embryo

The role of complement *in vivo* is not fully understood. It is needed in addition to antibody for bactericidal and hemolytic reactions of immune sera, as well as for other toxic effects (Boyd, 1956). Witebsky and Neter (1935) found that adding fresh guinea pig serum to heated rabbit antiserum against sheep red blood cells restored the toxic activity of the antiserum but not that of normal rabbit serum. Imagawa *et al.* (1954) showed that antisera produced in guinea pigs against mouse cancer cells when heated lost the ability to inactivate mammary cancer cells but that this activity could be restored by the addition of fresh guinea pig complement. Therefore, because previous experiments showed that complement was neither sufficient nor necessary for the toxicity of fresh rabbit serum, in an attempt to increase the effectiveness of the heated antiserum, complement was returned to the heated rabbit antiserum in the form of fresh guinea pig serum.

Fresh unheated guinea pig serum, obtained from the pooled blood of 4 to 6 guinea pigs, had no visible effects on the 72-hour chick embryo. Fresh guinea pig serum, when mixed with heated normal rabbit serum in varying proportions, also showed no visible effects. However, a mixture of guinea pig serum and heated antiserum against the 72-hour chick embryo was quite toxic to the 72-hour chick embryo, resulting in embryos in the group 5 condition (Table IV).

The effects of the addition of a second heat-labile substance, properdin, were examined next. Pillemer *et al.* (1954) found properdin in high concentration in the rat (25–50 units properdin/ml. serum), in intermediate concentration in the rabbit (4–8 units properdin/ml. serum), and in low concentration in guinea pig serum (1–2 units properdin/ml. serum). Therefore, rat serum was chosen as the source of properdin. Fresh rat serum was obtained from the pooled blood obtained by cardiac punctures from 4 to 6 large white rats. Fresh rat serum alone was highly toxic to the 72-hour chick embryo, producing the striking vascular phenomena described previously at all concentrations above 4%. Heating for 30 minutes at 56° C. removed all observable toxic effects. Preliminary experiments in which the embryos were examined 5 minutes to 4 hours after treatment showed that 4% fresh rat serum mixed with heated normal rabbit serum was extremely toxic to the 72-hour chick embryo.

In the case of rat serum, the titer of complement is low but the concentration of properdin was shown to be high, whereas, in the case of the guinea pig serum, the titer of complement is high but the concentration of properdin is low; therefore a study of the combination of rat and guinea pig serum, together with rabbit serum, was conducted. A mixture of fresh rat serum and guinea pig serum, at a dilution in which neither was capable of eliciting toxic effects alone, was quite toxic to the chick embryo. The toxicity of this mixture was also increased when heated normal rabbit serum was added to this mixture. The toxicity was lessened when the concentration of the heated normal rabbit serum was reduced by dilution with saline (1:2 to 1:4). These experiments suggest the possible interaction of heat-labile substances in guinea pig and rat sera with heat-stable substances in guinea pig, rat, and normal rabbit serum. Thus, the following absorption studies were conducted to remove nonspecific heat-stable substances.

Absorption Studies

Forssman antigen is reported to be present in the tissues of the chick embryo from the beginning of its development. Heated rabbit antiserum against sheep red blood cells mixed with guinea pig serum evoked the characteristic vascular phenomenon in the early chick embryo, whereas heated normal rabbit serum mixed with guinea pig serum would not (Witebsky and Neter, 1935). Therefore, it appeared imperative that Forssman type antibodies formed as a result of the injection of chick embryos into the rabbit be removed by absorption with chicken red blood cells (RBC). This procedure increased the specificity of the reaction but, owing to the concomitant dilution, decreased the sensitivity. When heated antiserum against 72-hour chick embryos was absorbed with chicken red blood cells at 37° C., the proportion of embryos showing the group 4 condition was decreased (Table V).

Adding fresh guinea pig serum increased the toxicity of the heated and absorbed antiserum. Several embryos in the group 5 condition were observed. Absorption of the fresh guinea pig serum with chicken red blood cells in the cold in the presence of Versene decreased the action of the heated and absorbed antiserum and absorbed guinea pig serum combination (Table V). This result may have been due to some inactivation of complement during the process of absorption.

As shown previously, fresh rat serum was extremely toxic to the 72-hour chick

embryo at concentrations above 4%. After absorption with chicken red blood cells in the presence of Versene in the cold (0 to 4° C.), the rat serum was no longer toxic to the embryo at concentrations below 10%. A mixture of 6% absorbed rat serum and 94% heated and absorbed rabbit antiserum was without effect on the embryo, as was a mixture of 6% rat serum and 94% heated and absorbed rabbit antiserum (Table VI). This is in strong contrast to the boosting effect of the addition of guinea pig serum to the heated rabbit antiserum. However, this finding is not unexpected, because properdin acts only in conjunction with complement and magnesium ions (Pillemer *et al.*, 1954) and the concentration of complement in rat serum is low (Hegedüs and Greiner, 1938).

A mixture of guinea pig serum and rat serum at a dilution in which neither could elicit toxic effects was shown to be extremely toxic to the 72-hour chick embryo. After absorption of the rat serum in the cold with RBC following filtration through a cation exchange (IRC-50) column, the toxic activity of the rat and guinea pig serum mixture was decreased.

TABLE V

The effects of absorption on the toxicity of heated rabbit antiserum against 72-hour chick embryos (HA72) and unheated guinea pig serum (GPS) combinations

Serum	Absorbed with	Combined with	No. embryos treated	Graded effects on embryos				
				1	2	3	4	5
HNRS	RBC	None	52	40	9	3	0	0
HA72	None	None	57	28	7	6	16	0
HA72	RBC	None	45	30	2	6	7	0
HNRS	RBC	GPS	19	18	1	0	0	0
HA72	RBC	GPS	63	5	7	8	3	40
HA72	RBC	GPS, heated	23	10	6	2	5	0
GPS	RBC	None	7	7	0	0	0	0
HNRS	RBC	Absorbed GPS	14	13	1	0	0	0
HA72	RBC	Absorbed GPS	17	3	3	1	4	6

The toxicity of a mixture of 6% fresh rat serum, 10% guinea pig serum, and 84% heated normal rabbit serum mixture was also reduced or removed altogether by the absorption of the guinea pig serum and rat serum at 0 to 4° C. with chicken red blood cells in the presence of Versene, and by the absorption of the heated normal rabbit serum at 37° C. with chicken red blood cells. On the other hand, a similar mixture of 10% absorbed guinea pig serum, 6% absorbed rat serum, with 84% heated and absorbed rabbit antiserum was toxic to the embryo. Immediate vascular effects were observed, followed by the cessation of heart contractions within 30 minutes (Table VI). The proportion of embryos exhibiting toxic effects was greater in this rabbit antiserum mixture containing both guinea pig serum and rat serum than that in rabbit antiserum mixtures containing either guinea pig serum or rat serum alone. Hence, a method is available to increase the effectiveness of antisera. Since rat serum enhanced the effect of the antiserum only in the presence of complement, it is suggested that a factor or factors analogous to properdin may be involved. Need for further experiments using purified properdin is indicated.

Properdin can participate in such diverse activities as the destruction of bacteria, the neutralization of viruses, and the lysis of certain red blood cells (Pillemer *et al.*, 1955). Although the presently reported experiments suggest the interaction of "the properdin system" with this specific antibody in the serum (A72), it is possible that the rat serum acts by supplementing the components of complement which are low in both rabbit and guinea pig sera, *e.g.*, the C'1 component (Hegedüs and Greiner, 1938). The application of quantitative techniques for handling complement and the use of purified components of complement, C'1, C'2, C'3, and C'4, may elucidate this aspect of the problem.

The absorption of the antiserum against 72-hour chick embryos with the homologous antigen removed most of the toxic effects of the antiserum on the 72-hour chick embryo. The antiserum was first heated and absorbed with chicken red blood cells in the manner described previously, and then mixed with a slight excess of minced and homogenized 72-hour chick embryos. The suspension was placed

TABLE VI

The effects of the addition of absorbed rat serum (RAT-RBC) and absorbed guinea pig serum (GPS-RBC) to heated and absorbed antiserum against the 72-hour chick embryo (HA72-RBC)

Serum	No. embryos treated	Graded effects on embryos				
		1	2	3	4	5
RAT-RBC (100%)	10	0	1	1	2	6
RAT-RBC (6 to 10%)	8	8	0	0	0	0
HNRS-RBC (94%) + RAT-RBC (6%)	9	9	0	0	0	0
HNRS-RBC (84%) + GPS-RBC (10%) + RAT-RBC (6%)	16	14	2	0	0	0
HA72-RBC (100%)	45	30	2	6	7	0
HA72-RBC (94%) + RAT-RBC (6%)	18	9	8	0	1	0
HA72-RBC (84%) + GPS-RBC (10%) + RAT-RBC (6%)	32	5	2	2	5	18

in the water bath for two to three hours at 37.5° C. A dense white precipitate was usually observed after 15 to 30 minutes. The tube was placed in the refrigerator at 0 to 4° C. for 12 hours and later centrifuged at 1200 RCF for 30 minutes. The supernatant was poured off into another tube containing a slight excess of homogenized 72-hour chick embryos. A smaller amount of precipitate was observed after the second absorption. The process was repeated until a negative interfacial "ring" test was obtained.

When guinea pig and rat sera were added to this heated antiserum which had been absorbed with both chicken red blood cells and 72-hour chick embryos, the toxic effects of the antiserum were not found to be completely removed (Table VII). The failure of the absorption of the antiserum by the homologous antigen is surprising but not without precedent. Ebert (1950) reported the failure of absorption by homologous antigen to remove the striking lethal and growth inhibitory powers of anti-organ sera. This non-absorption of one fraction of the antiserum was attributed to individual differences in the organ antigens used in injections

Table VII

The toxicity of heated antiserum against the 72-hour chick embryo (HA72) absorbed with the homologous antigen, singly, and in combination with absorbed guinea pig serum (GPS) and absorbed rat serum (RAT)

Serum	Absorbed with	No. embryos treated	Graded effects on embryos				
			1	2	3	4	5
HA72	RBC	11	8	1	0	2	0
HA72	RBC, 72-hour chick embryos	23	21	2	0	0	0
HA72 (90%) + GPS (10%)	RBC	57	3	5	7	3	39
HA72 (90%) + GPS (10%)	RBC, 72-hour embryos	34	21	6	2	4	1
HA72 (94%) + RAT (6%)	RBC	23	14	8	0	1	0
HA72 (94%) + RAT (6%)	RBC, 72-hour embryos	12	8	0	1	3	0
HA72 (84%) + GPS (10%) + RAT (6%)	RBC	32	5	2	2	5	18
HA72 (84%) + GPS (10%) + RAT (6%)	RBC, 72-hour embryos	14	7	1	1	1	4

and absorptions. Although large numbers of embryos were used in both injections and absorptions, a long course of injections was given. Such treatment often results in antisera of reduced specificity. This result may be even more pronounced in animals receiving adjuvant. However, the injection of adjuvant with heterologous antigen was insufficient to evoke a nonspecific response in the rabbit. Beef serum albumin (BSA) combined with adjuvant was injected into three rabbits. Tests of heated anti-BSA, and heated anti-BSA absorbed with BSA were negative (Table VIII). The number of different kinds of antibodies may be so great as to be incompletely absorbed by the antigen, even though an excess of antigen was used in absorptions and negative interfacial "ring" tests were obtained after the final absorption. This is not to say, however, that antibodies with new and different specificities are formed. The *in vivo* system employed here may be so sensitive as to respond strongly to these weaker or less "avid" antibodies. That embryonic proteins may be unique in their behavior in precipitin reactions was reported by Schechtman (1952), who found an unusual result when antiserum against the plasma from the 10-day embryo was reacted with adult and 10-day

Table VIII

Effects of antiserum against beef serum albumin (ABSA) on the 72-hour chick embryo

Treatment of serum	No. embryos treated	Effects on embryos				
		1	2	3	4	5
ABSA unheated	3	0	0	0	0	3
ABSA heated	7	6	1	0	0	0
ABSA heated and absorbed with RBC + GPS	15	15	0	0	0	0
ABSA heated and absorbed with RBC and BSA + GPS	6	6	0	0	0	0

serum. He wrote (p. 95), "This antiserum forms higher (antigen-antibody precipitation) curves with the heterologous antigen, adult serum. The antiserum is obviously not lacking in antibody since it produces heavy precipitates with adult material." He concluded that the embryonic serum forms antigen-antibody complexes with inferior light-scattering properties or that it contains substances inhibitory to the precipitin reaction.

Discussion

The toxicity of fresh rabbit serum to the early chick embryo was destroyed by heating at 56° C. for 30 minutes. The above experiments show clearly that the toxic substance in fresh rabbit serum is not complement; nor is it dependent on complement for its activity. In view of the latter observation, it is also probably not properdin. The following questions remain to be answered: (1) What are the physicochemical properties of this toxic heat-labile substance? (2) Is is composed of one or many substances? Can substances, other than complement, be separated or isolated from this heat-labile fraction which would further enhance the action of heat-stable fractions, as was shown above for complement and properdin or properdin-like substances? (3) What is the mechanism of action of this heat-labile substance? Is it similar to that brought about by heat-stable fractions? It was observed that the toxic effects of fresh normal rabbit serum in general resembled those produced by the action of heated rabbit antiserum to the 72-hour chick embryo coupled with fresh guinea pig serum and rat serum. Witebsky and Neter (1935) also described similar toxic effects on the early chick embryo of heated rabbit anti-sheep red blood cell serum plus complement. Pomerat (1949) reported similar results with rabbit anti-chick spleen serum. However, although the final picture appears to be the same, the mechanisms involved may not be similar. The development and use of more specific antisera to embryonic antigens may reveal more definitive and specific morphological expressions than those elicited by toxic factors in fresh rabbit serum. The present study has demonstrated that the 72-hour chick embryo is antigenic, *i.e.*, capable of eliciting the production of precipitating antibodies.

The presently reported investigation also demonstrated the fact that complement and properdin or properdin-like substances can play an active role in the action of the antiserum *in vivo*. The demonstrated ability of complement and properdin or properdin-like substances to increase the magnitude of the action of the antiserum will permit the observation of the effects of weaker but perhaps more specific antisera which otherwise would go unnoticed. Thus, the manner in which antisera act to block development or modify the normal function of reactive groups in the embryo may be studied more readily. The use of purified properdin or related substances, together with the components of complement, C'1, C'2, C'3, and C'4, may contribute to our understanding of the mechanism of action of the toxic antiserum *in vivo*.

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Summary

1. The specific objectives of the present investigation were at first two-fold: (1) to determine the antigenicity of the early chick embryo, and (2) to study the effects of homologous antisera on the chick embryo. However, because at the outset a profound toxic action of fresh normal rabbit serum was encountered, it became imperative to describe the toxic factor.

2. The toxic action of normal rabbit serum, characterized by the puckering of the blastoderm, the sinking of the embryo and its ultimate death, was removed by heating at 56° C. for 30 minutes. The toxic action was not restored by adding fresh guinea pig serum to heated rabbit serum. The toxicity was not removed by absorption in the cold with nonspecific antigen-antibody precipitates and/or chicken red blood cells. These results are interpreted as indicating that complement is neither necessary nor sufficient for the toxic action of fresh rabbit serum. The toxic heat-labile substance can also act independently of heat-stable substances which are removed by absorption with chicken red blood cells.

3. The antigenicity of the 72-hour chick embryo was demonstrated by its ability to elicit the production of precipitating antibodies in the rabbit. Heated rabbit antiserum against the 72-hour chick embryo evoked a weak but definite toxic response when placed on the homologous embryo.

4. In an attempt to decrease the probability of false negative reactions, methods were sought to increase the effectiveness of the antisera. Substances which may have been inactivated by heat were returned to the antiserum singly and in combination.

5. The toxic action of heated rabbit antiserum was partially enhanced by the addition of fresh guinea pig serum, rich in complement.

6. The toxic action of the heated rabbit antiserum was not increased by adding fresh rat serum, reported to contain large amounts of properdin, but was enhanced by a mixture of guinea pig serum and rat serum.

7. The results suggest the interaction of complement and properdin or a properdin-like factor in the action of the antiserum on the chick embryo.

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